Fluidic Sciences

Getting Started Guide

Protein labeling with Alexa Fluor™ NHS ester

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1. About this Getting Started Guide

This Getting Started Guide provides instructions for users who want to characterize proteins by Microfluidic Diffusional Sizing (MDS). The guide provides a generic protocol for labeling that yields products suitable for use on the Fluidity One-M platform and which can be easily adapted for any protein.

2. Protein labeling with NHS esters

NHS esters are amine reactive groups that can be used to covalently conjugate fluorophores to lysine residues in proteins (Figure 1). Since lysines are especially common residues on the surface of proteins, the use of NHS esters for labeling is straightforward and a widely used approach. The reaction of NHS esters with lysines depends strongly on pH. While at a too low pH, lysines are protonated and not accessible for conjugation, at a too high pH NHS esters get hydrolyzed reducing the amount that is available for conjugation. The optimal pH range for conjugation is 8.3 - 8.5.

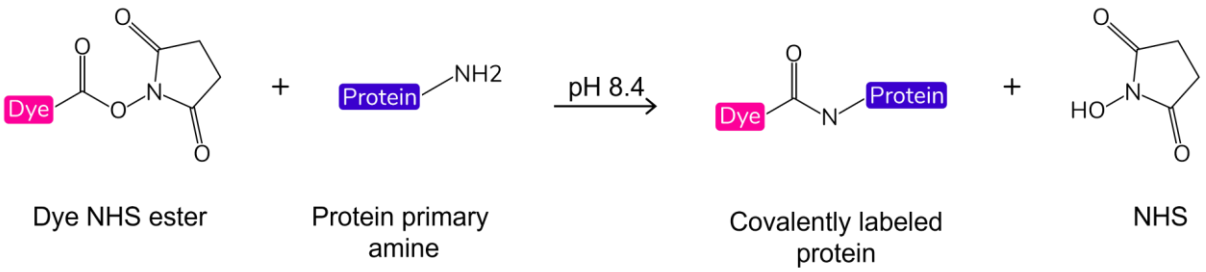

Figure 1. Schematic of amine labeling using NHS esters.

3. Required equipment, reagents and consumables

All reagents and equipment described in this Getting Started Guide have been validated for the Fluidity One-M system. While the use of reagents and equipment of similar quality is likely to achieve similar results, these have not been tested.

3.1 Required equipment

- Fluidity One-M (*Fluidic Sciences*)
- Nanodrop (*Thermo Fisher ND-ONE-W, or equivalent*)
- Pipettes (1000 µL, 200 µL, 10 µL; various suppliers)
- Zeba™ Desalting Chromatography Cartridges, 7K MWCO, 1 mL (*Thermo Fisher 89934*)
- pH meter (various suppliers)
- Centrifuge (*Eppendorf 5428000060,* or equivalent)

3.2 Required reagents

- Alexa Fluor™ 488 NHS Ester (*Thermo Fisher, A20000*) or Alexa Fluor™ 647 NHS Ester (*Thermo Fisher, A20006*)
- DMSO (anhydrous) (*Invitrogen, D12345*)
- 100 µg probe protein to be fluorescently labeled
- Sodium bicarbonate $NaHCO_3$ (*Merck S6014*)
- PBS buffer at pH 7.4 (*Merck P4417*)
- Tween® 20 (*Merck P7949*)
- Glycerol (*Sigma G9012*)
- Ultrapure water (various suppliers)
- HCl solution at 1 M for pH adjustment (various suppliers)

3.3 Required consumables

- Fluidity One-M chip-plate (*Fluidic Sciences*)
- 1000 µL, 200 µL, 10 µL low-retention pipette tips (various suppliers)
- Protein Lo-Bind tubes (*Eppendorf 0030108094* 0.5 mL; *Eppendorf 0030108116* 1.5 mL)
- Sterile filters, 0.22 µm, PVDF (various suppliers)
- 10 and 50 mL syringes, non-sterile (various suppliers)
- 5 mL Luer Lock syringe (e.g., *HSW HENKE-JECT®, Luer Lock 4050.000V0*, or equivalent)
- 1 mL Luer syringe (e.g., *HSW HENKE-JECT®, Luer 4010.200V0*, or equivalent)
- 50 mL Falcon tubes (various suppliers)
- Eppendorf rack (various suppliers)
- Disposable needle (various suppliers)

4 Protocol

4.1 Prepare 6-fold concentration labeling buffer

- Dissolve 0.84 g of sodium bicarbonate ($NaHCO_3$) in 9.5 mL of ultrapure water

Note: This might take 10 – 15 min depending on stirring speed and temperature

- Adjust pH to 8.3 with 1 M HCl if required
- Add ultrapure water to a final volume of 10 mL
- Filter using a pore size of 0.22 µm
- Store at -20 °C in 20 x 500 µL aliquots. If stored at -20 °C, 6-fold concentration labeling buffer can be used for up to 12 weeks

4.2 1% PBS-T (stock solution)

- Add 0.5 mL of Tween® 20 to 49.5 mL PBS and stir for at least 30 min at room temperature to obtain a stock solution of 1% Tween 20 in PBS pH 7.4
- Filter sterilize the solution using sterile filters (0.22 µm) and store at 4 °C
- 1% PBS-T can be used for up to 6 months

4.3 0.05% PBS-T (working solution)

- Mix 4.75 mL of filter sterilized PBS (pH 7.4) with 0.25 mL of 1% PBS-T stock solution
- Vortex for 10 sec
- Store at ambient temperature
- 0.05% PBS-T can be used for up to 8 weeks

4.4 Prepare Alexa Fluor™ label stock solutions

- Immediately before use, dissolve 1 mg of Alexa Fluor™ 488 NHS Ester or Alexa Fluor™ 647 NHS Ester in DMSO (155 or 80 µL respectively) to prepare a 10 mM solution; use a fresh, unopened vial of DMSO and do not re-use once opened
- Aliquot into 5 µL portions and store at -20 °C
- Labeled stock solution can be used for at least 12 weeks if stored at -20 °C
- Do not re-freeze aliquots once thawed

4.5 Labeling reaction

- Use 100 µg of probe protein at a minimum concentration of 0.5 mg/mL in PBS buffer at pH 7.4

Note: If the probe is dissolved in a buffer that contains primary amines (e.g., Tris-buffer), buffer exchange into PBS buffer (pH 7.4) is required prior to labeling

- Add labeling buffer as 6 x fold stock (i.e., 20 µL per 100 µL protein solution) and mix carefully by pipetting up and down 10 times. Do not vortex
- Add Alexa Fluor™ label stock solution at a molar ratio of 3:1 (label:protein) to the probe solution; refer to the Fluidic Sciences Labeling Calculator to determine required volumes

Note: As the label is usually dissolved in DMSO, check that the final concentration of the label stock solution in the labeling reaction does not exceed 1.8 % to prevent DMSO interfering with the structural integrity of the probe.

- Carefully pipette up and down 10 times; do not vortex

- Incubate the labeling reaction at 4 °C overnight protected from light

4.6 Purification

If an ÄKTA or other FPLC system is available, the highest quality reagents can be achieved by purifying the labeled antigen using SEC according to the recommended protocol of the system and column used. If purification by SEC is not possible the following quick benchtop protocol is suitable for most antigens with a molecular weight above 20 kDa:

- Connect a 5 mL Luer Lock syringe to a desalting column and equilibrate with 5 mL of 0.05% PBS-T (pH 7.4)
- Place 10 Eppendorf tubes in a rack, leave the lids open
- Connect a disposable needle to a 1 mL Luer syringe
- Draw the labeling mixture into the 1 mL Luer syringe
- Remove the needle from the syringe and dispose appropriately
- Remove trapped air from the syringe before connecting it to the desalting column
- Push the sample onto the column

Note: It is not necessary to collect the flow-through at this stage

- Fill a second, unused 1 mL Luer syringe with 1 mL of 0.05% PBS-T (pH 7.4) buffer
- Elute the sample by collecting fractions of approximately 100 µL in the prepared Eppendorf tubes. Read the fraction volumes using the scale of the syringe
- The protein will likely elute in fractions 2 – 4, and later eluting fractions will likely contain unbound label

4.7 Labeling QC

- Measure yield of labeled probe as well as labeling ratio
    - To determine the yield as well as labeling ratio, measure the absorbance of all fractions at a wavelength of 280 nm and the absorbance of the conjugated label on a Nanodrop (select either Alexa Fluor™ 488 or Alexa Fluor™ 647 depending on which dye was used)
    - Disable sloping dye correction and Analysis correction functions on the Nanodrop

Note: If using a different model of UV/Vis spectrometer the measured absorbance needs to be corrected manually. Correction factors are 0.11 and 0.03 for Alexa Fluor™ 488 and Alexa Fluor™ 647 respectively.

The following equations yield the corrected protein concentration (required only when not using the automated correction on a Nanodrop).

Alexa Fluor™ 488:

$$\text{protein concentration (M)} = \frac{[A_{280\ \text{nm}} - 0.11 \times A_{494\ \text{nm}}] \times \text{dilution factor}}{\text{molar extinction coefficient at 280 nm}}$$

Alexa Fluor™ 647:

$$\text{protein concentration (M)} = \frac{[A_{280\ \text{nm}} - 0.03 \times A_{650\ \text{nm}}] \times \text{dilution factor}}{\text{molar extinction coefficient at 280 nm}}$$

- The labeling ratio should be between 0.5 and 2 dye molecules per protein
    - If considerably higher label-to-probe ratios are measured (e.g., >3), unbound label was not completely removed, and the desalting procedure should be repeated

- Check the R_h of the fluorescently labeled probe on a Fluidity One-M
    - Dilute the labeled probe to a concentration of 50 nM in a volume of 15 µL using PBS-T buffer (pH 7.4)
    - Measure the size of the purified labeled protein in triplicate on a Fluidity One-M as instructed in the user guide.
- The measured Rh should fall within ±10% of the expected value (consult the Fluidic Sciences hydrodynamic radius calculator)
    - If an R_h lower than expected is measured, unbound label was not completely removed, and the desalting procedure needs to be repeated
- Typical yields of labeled probe are ≤50 µg
- Store the labeled probe at an appropriate buffer condition and temperature

Note: Use of a cryoprotectant (e.g., 10% glycerol) and flash-freezing in liquid nitrogen is recommended for storage at -80 °C

About us

It's not just the proteins that make life, it's the interactions among them. Here at Fluidic Sciences, we make protein interaction analysis easy and robust by developing transformative in-solution technologies and accessible instruments that help scientists quickly and accurately understand how proteins truly interact.

For more information about us, please visit our website www.fluidic.com.